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Poly(A) Tail Metabolism and Function in Eucaryotes*

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Recent work on the homopolymeric poly(A) tail of eucaryotic mRNA has led to a clearer understanding of its synthesis, degradation, and function. This review will emphasize the key insights of these studies and attempt to summarize current working models. Two recurring themes in poly(A) research, the recruitment of enzymes by RNA binding proteins and the recognition of degenerate RNA sequences by combinations of RNA binding proteins, will also be highlighted.

Poly(A) Metabolism

Nuclear Polyadenylation—The 3′-ends of mRNA are generated in the nucleus in two steps. The precursor RNA, extending beyond the mature 3′-end for hundreds of nucleotides, is cleaved endonucleolytically at one particular phosphodiester bond. The 3′-OH group of the upstream fragment then receives the poly(A) tail by polymerization from ATP. The cleavage reaction depends on two RNA sequence elements, the highly conserved sequence AAUAAA 10–30 nucleotides upstream of the cleavage site and a poorly defined GU- or U-rich sequence approximately the same distance downstream. In some cases, sequences upstream of AAUAAA increase the efficiency of 3′-end processing. *In vitro*, polyadenylation can be carried out independently of cleavage with precleaved RNA substrates ending at the cleavage/polyadenylation site. This reaction also depends on the sequence AAUAAA (1).

AAUAAA is the binding site for the cleavage and polyadenylation specificity factor (CPSF),[1] which is, like AAUAAA, essential for both steps of the processing reaction. CPSF contains subunits of 160, 100, 73, and, possibly, 30 kDa (2). The largest subunit may have RNA binding activity (3). The sequence element downstream of the cleavage/polyadenylation site appears to be bound by a protein known as cleavage stimulation factor (CStF) or cleavage factor I (CFI) with subunits of 77, 64, and 50 kDa (4, 5). The 64-kDa subunit can be UV-cross-linked to RNA, and its cDNA sequence shows a ribonucleoprotein-type RNA-binding domain (6). CStF/CFI is not involved in polyadenylation.

One or two additional factors, required to reconstitute specific 3′-end cleavage, have not been purified so far. Like CStF/CFI, they are dispensable for polyadenylation. Finally, poly(A) polymerase, the enzyme that synthesizes the poly(A) tail, is also involved in the cleavage reaction. Initially purified as a fully active degradation product of 60 kDa (7), it has been shown by cDNA cloning to exist in at least two forms of 78 and 83 kDa (8, 9).

Prior to the cleavage reaction, processing factors and substrate RNA form a large complex that is detectable after incubation of the RNA in crude nuclear extract with ATP. As the complete set of processing factors is not yet available in pure form, only partial complexes have been reconstituted with purified material. CPSF by itself binds to RNA containing the AAUAAA sequence (3). The CPSF·RNA complex is quite unstable; it is stabilized by CStF/CFI (4, 10). Since this stabilization requires the downstream sequence element, one may infer that CStF/CFI binds to this sequence. Binding of CStF is also enhanced in the ternary complex with CPSF as judged by a more efficient cross-linking of its 64-kDa subunit (11). Binding of CPSF to RNA is also strengthened by poly(A) polymerase, possibly explaining why this enzyme stimulates the cleavage reaction (12). From these data one may infer that the 3′-processing complex contains, in addition to the RNA, at least CPSF, CStF/CFI, and poly(A) polymerase. Unresolved questions include the roles of the still uncharacterized cleavage factors, the identity of the endonuclease, and the role of ATP in complex formation and cleavage.

Once cleavage has occurred, polyadenylation ensues in a closely coupled fashion; the cleaved intermediate is not detectable under normal reaction conditions. The tight coupling is probably due to the presence of poly(A) polymerase in the cleavage complex. With a precleaved substrate, CPSF and poly(A) polymerase are sufficient for poly(A) synthesis. In the absence of CPSF, poly(A) polymerase is not only almost completely inactive but also indifferent to the presence or absence of the AAUAAA sequence in the substrate RNA. The inactivity reflects a poor affinity for RNA; complexes between poly(A) polymerase and RNA can be detected only at high protein concentrations. A ternary complex containing poly(A) polymerase, CPSF, and the substrate RNA can be inferred from its increased stability compared with a CPSF·RNA complex (see above) and its decreased mobility in gel shift experiments. Thus, CPSF recruits poly(A) polymerase to the RNA and makes it specific for RNAs that contain the CPSF binding site, AAUAAA (12).

Polyadenylation by purified poly(A) polymerase and CPSF is strongly stimulated by poly(A) binding protein II (PABII) (13, 14). The nuclear PABII is different from the cytoplasmic poly(A) binding protein I (see below). Once the growing poly(A) tail has reached a length of 10–12 nucleotides, PABII binds and the further rate of extension is strongly increased. Poly(A) synthesis is therefore biphasic, with a slow oligoadenylation phase followed by rapid extension. In the presence of PABII, RNAs that carry an oligo(A) tail capable of binding PABII can be extended independently of CPSF and its binding site AAUAAA. However, extension is much more rapid when both stimulatory factors are present. PABII also stimulates 30–50-fold the extension of a simple poly(A) primer in the absence of CPSF. Like CPSF, PABII appears to act by recruiting poly(A) polymerase to the RNA. A complex containing PABII and poly(A) polymerase on the RNA has not yet been directly demonstrated. However, PABII further stabilizes the complex containing PAP and CPSF, suggesting the existence of a quaternary complex (12).

Changes in the functional properties of poly(A) polymerase are consistent with the hypothesis of quaternary complex formation. On its own, the enzyme is entirely distributive, dissociating from its primer after every polymerization event. In the

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[1] The abbreviations used are: CPSF, cleavage and polyadenylation specificity factor; CStF, cleavage stimulation factor; CFI, cleavage factor I; PABII, poly(A) binding protein II; PAN, poly(A) ribonuclease; UTR, untranslated region; CPE, cytoplasmic polyadenylation element.

presence of either PABII or CPSF, the processivity increases slightly to less than 10 nucleotides polymerized per binding event. Only in the presence of both stimulatory factors can poly(A) polymerase synthesize a full-length poly(A) tail of 200 nucleotides in a single processive event (12).

In the nuclei of mammalian cells, poly(A) tails grow to a length of 200–250 nucleotides before the RNA is exported to the cytoplasm. The *in vitro* system reconstituted from purified CPSF, poly(A) polymerase, and PABII reproduces this length control; processive synthesis stops after the addition of approximately 200 adenylate residues, and only slow, presumably nonprocessive synthesis continues (13). The mechanism inducing this loss of processivity is not understood.

In summary, the apparently simple reaction of cleavage and polyadenylation is carried out by a surprisingly complex set of at least five factors, several of which are composed of multiple subunits. Most of these factors are incorporated into a large complex with the substrate RNA prior to the first catalytic step. Several aspects of this complex may be relevant to other RNA processing reactions. For instance, many factors serve in substrate recognition rather than catalysis. Substrate recognition is achieved through a network of weak interactions rather than by a few high affinity interactions. Such a requirement for the colocalization of many independent factors provides a possible explanation for why mRNA processing signals can be both large in size and degenerate. Similarly, the interactive fashion of substrate recognition also suggests that the requirement for a certain part of a processing signal or a certain factor may depend on the "strength" of the remaining parts of the signal or the concentration of all other factors. Finally, the conventional idea of a linear reaction pathway may not always be appropriate to describe the assembly of a processing complex. Although many binding reactions influence each other, there is no strict dependence of one on the other. Thus, several alternative pathways may lead to the same product, and a preferred pathway established for one substrate under one set of conditions may not hold for a different substrate or a different set of conditions.

Cytoplasmic Deadenylation—Poly(A) tail shortening in the cytoplasm is initiated after nuclear transport, although it is not known if this reaction first requires the association of the mRNA with the translational apparatus (15). Insight into the mechanism of deadenylation was achieved with the observation that deletion of the cytoplasmic poly(A) binding protein (PAB1) from yeast prevented the normal shortening of the cellular poly(A). This result was in direct contrast to the anticipated one of induced tail destabilization since it had been previously thought that mRNA binding proteins protect the RNA from nucleases. Instead, these data suggested that a deadenylase in yeast required PAB1 for function (16).

The yeast poly(A) ribonuclease (PAN) was purified from soluble extracts based upon its ability to degrade poly(A) only in the presence of PAB1 (17). The purified enzyme displays an absolute requirement for a ribonucleoprotein substrate. PAN is normally a distributive exoribonuclease that releases 5′-monophosphates in a Mg^{2+}-dependent reaction. Poly(A) is degraded in two kinetically and biochemically distinct phases. In the shortening phase, PAN degrades poly(A) down to a minimal length of 12–25 adenine residues, a length sufficient to bind one PAB1 molecule (18). Interestingly, PAN can shorten some substrates in the absence of PAB1 as long as an appropriate concentration of spermidine is present. In the terminal deadenylation phase, PAN brings the mRNA to a completely deadenylated form. This second reaction can also occur in the absence of PAB1 at a much slower rate. The temporal separation of the shortening from the terminal deadenylation reaction probably results from the inability of PAB1 to slide or dissociate from the residual poly(A) tail. A recently described mammalian poly(A) nuclease also exhibits a Mg^{2+}-dependent exonucleolytic activity that releases 5′-monophosphates (19). Whether this enzyme requires a ribonucleoprotein substrate for activity is not yet known.

Many similarities exist between the mechanisms of polyadenylation and deadenylation. First, each of the reactions occur in two phases. Second, each of the reactions have a dependence on a poly(A) binding protein that explains their biphasic nature. Finally, each of the PABs is required for the reactions to occur efficiently, suggesting that a key function for the PABs in each of these reactions is the localization of either poly(A) polymerase or PAN to their substrate.

Poly(A) Metabolism in the Oocyte—Animal oocytes contain large amounts of mRNA stored for use during maturation and early embryonic development. Gene expression during these stages can be regulated at the level of translation, and this occurs in many cases through alterations in the poly(A) tail lengths. Tail length is regulated by the control of both its synthesis and degradation (for a more detailed review of the following topics see Ref. 20).

Messenger RNA synthesized in the oocyte before maturation is polyadenylated normally in the nucleus. In the cytoplasm, it is subject to apparently two different deadenylation reactions. Maternal transcripts being translated retain poly(A) tails longer than 50 nucleotides. In contrast, transcripts that are not being translated in the oocyte but which will be subject to regulated polyadenylation and translation following oocyte maturation are deadenylated to an even greater extent. The specific deadenylation of these transcripts appears to result from the presence of a unique sequence in their 3′-UTRs (21).

Following maturation, the translational pattern of the oocyte changes. Some previously active mRNAs are no longer translated as a result of their deadenylation by a potentially different enzyme than that found in the immature oocyte. This poly(A) ribonuclease is activated only after the oocyte's cytoplasmic and nuclear compartments merge due to the maturation-induced germinal vesicle breakdown. This ribonuclease activity does not require specific signals. For instance it will destroy polyadenylated bacterial sequences or even polyadenylated poly(C).

Maturation also induces the translation of previously dormant RNAs, and these are subject to an extension of their poly(A) tail. This developmentally regulated polyadenylation occurs in the cytoplasm, in contrast to the nuclear 3′-processing reactions. The reaction depends upon a sequence related to UUUUUUAU, the cytoplasmic polyadenylation element (CPE), and it also requires the AAUAAA polyadenylation signal. RNAs that have both a CPE and AAUAAA receive a poly(A) tail upon oocyte maturation; all others lose their tail. Polyadenylation thus seems to be a positively regulated event overriding the default deadenylation reaction. The implication of this is that changes in poly(A) tail lengths in the developing organism result from regulated adenylation and not deadenylation. However, the possibility still exists that the deadenylation of some mRNAs is induced by a unique deadenylation element.

CPE-dependent polyadenylation can be carried out in *Xenopus* egg extracts. Their fractionation demonstrated a poly(A) polymerase activity as well as an RNA binding activity specific for both AAUAAA and the CPE. It is the RNA binding activity that is induced by oocyte maturation (22). In contrast, poly(A) polymerase activity is similar before and after maturation. Surprisingly, CPE-dependent polyadenylation can be reproduced *in vitro* with recombinant mammalian poly(A) polymerase and purified mammalian CPSF. Direct experiments suggest that CPSF has a binding specificity not only for AAUAAA but also

for a CPE-like sequence.[2] Thus, it is possible that cytoplasmic polyadenylation is catalyzed by essentially the same components as the nuclear reaction. Additional factors may modify the basic reaction. It is also possible that the sequences upstream of AAUAAA, which influence the polyadenylation of a number of genes in mammalian cells, function like the CPE in enhancing the binding of CPSF.

Poly(A) Function

Understanding the function of the poly(A) tail remains a major goal. Significant progress in two areas, mRNA degradation and translation initiation, has shed light on the role of the polymer and highlighted a fundamental theme in RNA metabolism, that of ribonucleoprotein recognition by trans-acting factors. Areas that remain less well defined but which could also require the poly(A) tail are intracellular mRNA localization and mRNA nuclear transport.

mRNA Degradation—The destruction of eucaryotic mRNA is a highly regulated process that occurs at rates differing by over 2 orders of magnitude (for a detailed review, see Ref. 23). The information that determines at what rate an mRNA is to be destroyed lies within destabilizing determinants throughout the message. While examples of destabilizing sequences abound, the only sequences known to stabilize mRNA directly are the cap structure and the poly(A) tail. It is this property of stabilization that makes poly(A) tail removal a target for the activity of the destabilizing sequences elsewhere on the mRNA.

That poly(A) tail removal occurs before mRNA is degraded has been shown for several different messages with essentially the same technique (23). In these experiments, mRNA synthesis is initiated and then abruptly inhibited. The bolus of RNA that is produced at first carries the long nuclear poly(A) tail, and once transported to the cytoplasm it begins to lose its tail. The degradation of the mRNA is found to begin only after the poly(A) tail has been removed. For example, mRNAs that are rapidly degraded first lose their tails rapidly, while those that are slowly degraded first lose their tails slowly. Furthermore, mutations that alter the rate of mRNA deadenylation also change the rate of degradation.

Each of these experiments has contributed to the working model that destabilizing sequences function by stimulating deadenylation. This stimulation can be for either the shortening or terminal deadenylation phase, or both. A closer examination of the available data suggests that the mRNA need not be completely deadenylated before the next phase of the degradation reaction occurs (24). Instead, it seems that shortening of the oligoadenylate tail to a length below approximately 10 nucleotides, a length incapable of high affinity binding to PAB1, is sufficient to induce the next step. Whether the next step in the decay reaction is also common to all mRNAs or whether it is at this juncture that each mRNA is destroyed by separate pathways remains to be determined.

3′-UTR-dependent deadenylation utilizing the purified yeast PAN has been reconstituted, providing some mechanistic data about the action of these elements (18). For instance, it is clear that *in vitro* the terminal deadenylation step occurs at different rates for different mRNAs and that the determinants for these rates can lie within the 3′-UTR of the mRNAs. Furthermore, the identification of an mRNA sequence that completely inhibits terminal deadenylation *in vitro* raises the possibility that mRNA can be stabilized *in vivo* by similar sequences.

Finally, *in vitro* deadenylation studies on the yeast MFA2 3′-UTR reveal that it stimulates deadenylation of the mRNA by inducing PAN processivity (18). This simple switch of enzyme mechanism has important implications for studying the mode of action of the destabilizing elements. It provides support for the simple model that all destabilizing sequences operate by targeting association factors for PAN to the mRNA. As a result, biochemical dissection of the mechanism of action of these elements could utilize changes in PAN activity as an enzymatic assay. In general, the regulation of mRNA decay by changes in PAN processivity is a recapitulation of the recurring theme of differential affinity of enzymes for their substrate induced by ribonucleoprotein complexes.

Translation Initiation—Subsequent to the discovery of the poly(A) tail, the potential role for this sequence in mRNA translation was explored and then dismissed based upon the data that poly(A)-deficient mRNA was translated *in vitro* almost as well as polyadenylated mRNA (reviewed in Ref. 25). However, based on the recent observations summarized below, it is now generally accepted that mRNA translation is most efficient with, and sometimes even completely dependent upon, the poly(A) tail (for a more detailed treatment, see Ref. 26).

Investigations into the cytoplasmic polyadenylation and deadenylation reactions in the maturing oocyte support the hypothesis that the purpose of controlling the adenylation status of the mRNA is to control its translatability (reviewed in Ref. 20). Mutations that destroyed the CPE on a maternal mRNA prevented both its polyadenylation and translation. Furthermore, if an mRNA that was normally deadenylated and translationally repressed during maturation had inserted within its 3′-UTR a CPE, it remained polyadenylated as well as translationally activated. Proof that the translational recruitment was specific for the poly(A) tail and not the presence of a CPE came with the observation that a CPE containing mRNA modified with a dideoxyadenosine at its 3′-end was neither translationally competent nor capable of cytoplasmic polyadenylation. Finally, for several mRNAs, *in vitro* polyadenylation followed by oocyte injection was sufficient to induce translation at an inappropriate time.

Independent genetic data from the yeast *Saccharomyces cerevisiae* also support the conclusion that poly(A) is necessary for translation *in vivo* (reviewed in Ref. 15). In these experiments, temperature-sensitive mutations in the essential yeast *PAB1* gene were found to cause an inhibition of translation at the restrictive temperature. The accumulation of ribosomal subunits at this temperature indicated that the inhibition of translation was due to a block in initiation. Support for this hypothesis came from an examination of suppressors of a *PAB1* deletion. Each of these suppressor mutations decreased the level of the 60 S ribosomal subunits. One of the mutations resulted in the loss of the large ribosomal subunit protein L46, while another altered a polypeptide with significant homology to a family of RNA-dependent ATPases that appeared to be involved in ribosomal RNA maturation. These data confirmed the conclusion that the primary deficiency in *pab1* mutants was translational and that a 60 S subunit-dependent step in the translation initiation pathway was affected.

Further support for this conclusion came from an analysis of the mild stimulation of translation *in vitro* by poly(A) (27). This study found that the 60 S subunit joining step was more efficient when the mRNA was polyadenylated. More recently, genetic interactions between the yeast SIS1 protein required for translation (28) and PAB1 have enlarged the list of potential players mediating the translational requirement for the poly(A) tail. Finally, the discovery that PAN, the PAB1-activated ribonuclease, is also required for translation initiation (17) creates a new series of questions regarding the relationship between the mRNA degradation and translation initiation reactions.

[2] A. Bilger, C. Fox, E. Wahle, W. Keller, and M. Wickens, manuscript in preparation.

Summary and Perspective

The detailed examination of poly(A) tail synthesis, degradation, and function has yielded important insights. Enzyme recognition of ribonucleoprotein substrates and alterations in enzyme mechanism due to variations in substrate affinity are two important examples. The utilization of the poly(A) tail in multiple reactions shows that a single RNA sequence can be recognized by different groups of enzymes. The requirement for poly(A) in each of these reactions explains why sequence elements within mRNA that control poly(A) tail metabolism have such a dramatic effect on mRNA expression.

Future work in poly(A) research will almost certainly include a more detailed analysis of both the adenylation and deadenylation reactions, the identification of more sequence elements that can regulate them, and the reconstitution of many of the poly(A)-dependent events within the cell. With this should come an even greater appreciation for the mechanisms used in the post-transcriptional control of gene expression.

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